THE EMBO JOURNAL

Manuscript EMBO-2014-90493

Dysregulated miRNA biogenesis downstream of cellular stress and ALS-causing mutations: a new mechanism for ALS

Anna Emde, Chen Eitan, Lee-Liong Liu, Ryan T Libby, Natali Rivkin, Iddo Magen, Irit Reichenstein, Hagar Oppenheim, Raya Eilam, Aurelio Silvestroni, Betty Alajajian, Iddo Z Ben-Dov, Julianne Aebischer, Alon Savidor, Yishai Levin, Robert Sons, Scott M Hammond, John Ravits, Thomas Müller & Eran Hornstein

Corresponding author: Eran Hornstein, Weizmann Institute of Science

Review timeline:

Submission date:	09 November 2014
Editorial Decision:	08 December 2014
Revision received:	12 May 2015
Editorial Decision:	09 June 2015
Accepted:	20 July 2015

Editor: Karin Dumstrei

Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 08 December 2014

Thank you for submitting your manuscript to The EMBO Journal. Your manuscript has now been reviewed by two good experts in the field and their comments are provided below.

As you can see, both referees appreciate the core finding that microRNAs expression is downregulated in ALS. However they also find that the follow up analysis needs more work and that the connection between stress granules and miRNA processing needs to be better delineated. The statistical analysis also needs to be better worked out. The referees provide very constructive comments and I suspect that you will be able to resolve many of the concerns raised. Should you be able to address the concerns raised by the referees then I would like to consider a revised version. I should point out that it is our policy to allow for one major round of revision only and that it is therefore important to address the major concerns at this stage.

Since significant revisions are needed, I think it would be most productive if we discussed the revisions upfront. It would be helpful if you could send me a point-by-point response outlining what can be done within a reasonable timeframe and then we can discuss the revisions further.

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process, please visit our website: http://emboj.embopress.org/about#Transparent_Process

We generally allow three months as standard revision time. As a matter of policy, competing manuscripts published during this period will not negatively impact on our assessment of the conceptual advance presented by your study. I can also extend the revision deadline if needed.

Thank you for the opportunity to consider your work for publication. I look forward to hearing back from you.

REFEREE REPORTS

Referee #1:

Emde et al find evidence for altered microRNA processing in motoneurons of ALS patients and in ALS in vitro systems. Pharmacological enhancement of miRNA processing restores some of the phenotypes, but does not prolong the life of SOD1 mice. The changes in miRNA levels upon various genetic and pharmacological manipulations are attributed to stress granule (SG) formation, but stress granules are never directly analyzed throughout the whole manuscript. The authors postulate an interesting new hypothesis and show data in various model systems (including an impressive two mouse models). On the one side, it is nice that miRNAs seem to be affect different ALS models, on the other side there is no red thread throughout the manuscripts. The authors switch between genetic and pharmaceutical induction of different kinds of cellular stress and analyze the effect on a changing set of miRNAs. Several connections are implied, but not firmly shown. To justify publication in the EMBO journal the mechanistic links between ALS mutations, stress granules and miRNA processing needs to be carved out more carefully.

Specific points:
Fig 1A: The global suppression of microRNA expression is stunning. The effect depends on the validity of two reference genes. The authors should show qPCR data for other unrelated small RNAs (e.g. snoRNAs) normalized to the same reference RNAs. It would be nice to see global changes in expression of pre-miRNA and mature miRNAs by P32 5' end-labeling.
Fig 1 E/F: Are the miRNA changes in sALS and fALS correlated? The raw data for Fig 1 should be included as a table.
Fig 2: Why were these miRNAs selected? Why are different miRNAs shown in the different panels. Do different stressors affect different miRNAs? If so, how? The legend (grey vs. black) should be clearly shown in all graphs
Fig 2j/k: Quantification of several replicates needs to be shown. Fig 2k is not mentioned in the text. Are Dicer levels reduced by arsenite treatment?
Fig 2L: What's known?
Fig 2L: Puromycin inhibits overall translation. Are the protein levels of the Dicer complex components reduced to directly inhibit Dicer activity independent of stress granules? Compare slight effect on Dicer by arsenite treatment in Fig. 2K. The authors claim the change in miRNAs is due to induction of stress granules. The authors should show that puromycin really induces stress granules within the 24h by immunofluorescence (ideally time course experiment).
Fig 3A: Does FUS R495X expression induce cellular stress and cause formation of stress granules in this model? In most publications mutant FUS localizes to SGs only after stress induction with arsenite or heat shock (e.g. Bosco et al 2010, Dormann et al 2010, Daigle et al 2013) but not by itself. The discrepancy may be due to different stress levels induced by the various transfection protocols. The authors should confirm that only transfection of mutant FUS does indeed induce SGs under their conditions using immunofluorescence with marker antibodies. They should also show the effect of mutant FUS compared to wildtype FUS on at least on the most regulated miRNAs. Also, why are some miRNAs more affected? Does this reflect a different half-life time?
Fig 3C: It seems that mutant FUS reduces Dicer protein levels. Please show quantification.
Fig 4A: The authors need to show that mutant protein expression indeed induces SGs specifically compared to wt proteins by IF and quantify miRNA changes also compared to wt control. Especially overexpression the wildtype RNA-binding proteins TDP-43 and FUS could by itself affect miRNA levels. Moreover, they have to show that CHX treatment inhibits SG formation and not merely reduces expression of the mutant proteins. I don't know the half-life time of these proteins, but I could imagine that 72h CHX treatment would dramatically reduce expression levels of the transfected proteins. This would severely confound interpretation of this rescue experiment. What

are the effects of CHX on miRNA levels in untransfected cells?
Fig 4B,C: The authors should validate their key findings by western blot.
Fig 4D,E: These experiments should also be performed with wt control.
Fig 4A,F and 5: error bars missing
Fig 5: error bars missing
Fig 6A/B: I would prefer to see how the miRNAs and mRNAs levels are changing in the control mice of time instead of normalization to 1.
Fig 6C: Are these the most dysregulated miRNAs in SOD1 animals?
Fig 6D: How the expression levels of miRNA processing machinery in SOD1 mice compared to nontransgenic littermates?
Fig 7F,G,H,J: No post-test was done in the ANOVA. Are the relevant pairs not significantly different?
Fig 7i: Here a post-test was performed, but the relevant pairs should be marked in the graph.
Fig 7j: Does a lower hangwire score indicate greater strength or were the labels swapped?
Fig 7K: How are two curves compared by a t-test?

Referee #2:

In the present manuscript, Emde et al. explore a potential role of impaired pre-miRNA processing in ALS. They show that stress and ALS mutations lead to altered pre-miR/mature miR levels for some miRNAs, possibly by affecting the composition of the Dicer processing complex and eIF2alpha signaling. Treatment of mice with an enhancer of miR processing, Enoxacin, provides benefitial effects for some neurological aspects of ALS. Invoking altered miR processing in ALS is a novel and interesting hypothesis, and the elucidation of such a mechanism could provide a novel avenue for ALS treatment. However, the data presented in this manuscript is in most cases at best preliminary and largely does not support the claims of the authors. Before this manuscript can be published, it has to be extensively revised, i.e. by completing preliminary datasets, by providing new experiments that functionally link stress-dependent remodeling of the Dicer complex to altered miRNA biogenesis in ALS and by providing a sound statistical analysis. In addition, the authors should pay attention to my detailed comments provided below.

General comments:

1. The data presentation, in particular graphs supposed to show altered pre-miRNA processing, are very confusing. Why are the authors not presenting individual bars for pre-miRNAs and miRNA levels under different treatment conditions, instead of ratios?
2. The statistical analysis is completely insufficient. Some panels lack error bars entirely, why in others different time points are grouped together to achieve statistical significance. Moreover, statistical methods are not presented in the figure legends, making an independnet assessment oft he data impossible. For example, often single bars are marked with asterisks, without any indication to what other value(s) the comparison was actually made (e.g. Fig. 2, 4). If the data were normalized, how could a statistical significance actually be calculated?
3. The rationale for choosing miRNA candidates is not provided. Were these the miRs which displayed the most pronounced differences? There is also quite some discrepancy regarding the results between different cellular models (i.e. HEK cells vs. Motoneurons), but this point was not addressed.
4. Although altered pre-miR/miRNA ratio correlate with changes in the composition of the Dicer complex, there is actually no data that definitely shows reduced Dicer activity. One solution would be to perform in vitro Dicer processing assays with extracts obtained from the different models. This would also allow to deplete/substitute components of the Dicer complex to interrogate their potential contribution to altered pre-miR processing.
5. Overall, the recovery obtained with Enoxacin treatment appears to be rather modest, and only a few specific miRNAs seem to be affected (Fig. 5C). There is also a disconnect in that Enoxacin targets the Dicer-TRBP interaction, which is not affected by stress (Fig. 4C). This raises the question whether enoxacin is in fact the right drug to improve miRNA processing in ALS.

Specific concerns:

2A-I: The presentation of pre-miR vs. Mature miR is very confusing. The authors should show

values for pre-miR and mature miR for both control and experimental condition. How are the p-values actually calculated? This should be clearly indicated in the figure legends. There is also no discussion how miR candidates were selected, and why the processing of only a specific subset is significantly affected. In this context, the authors should tone down some of their statements regarding global effects on miR processing.

2K: From this blot, it looks like Dicer is strongly downregulated upon stress, but this observation is neither discussed nor followed up. It would also be important to show the full blots to see other Dicer isoforms that have been reported.

2L: what is this figure supposed to show? Most of the data is quite variable and differences do not reach statistical significance. Moreover, puromycin clearly affects processes other than stress granule formation, which should be discussed.

3A: Now, even a different form of data presentation compared to Fig. 2 is chosen, which leads to further confusion. The fact that a substantial fraction of miRs actually shows reduced pre-miR/miR ratios is not discussed.

4A: These panels lack error bars or p-values, which makes it impossible to judge the reproducibility of the experiments. Since cycloheximide is a blocker of translation, other translation blockers should be used for comparison, e.g. emetine.

4B,C: It would be important to show representative blots. Since these experiments are done in HEK cells, which show quite different effects on miR processing compared to motoneurons, the relevance of these findings remains somewhat unclear. As mentioned above, it is quite puzzling that the major regulatory proteins of Dicer, TRBP and PACT, are not affected by stress.

4D: again, the presented changes are apparently not significant, so what is this figure supposed to show? Statistical test is not indicated.

4F: see my comments to Fig. 4A

5: see my comments to Fig. 4A and F.

6A, B: now, a different panel of miRs was interrogated. I found it quite unusual that significance was calculated over different time points, so this should be complemented by a statistical assessment of individual time points. Also, the test used should be indicated.

6C: This figure actually shows that enoxacin has only very modest effects on the relative expression of miRs, and that only a minority of miRs is affected. This should be clearly stated in the text. In addition, an important control experiment (control vs. Control enoxacin) that addresses the effects of enoxacin in control animals was omitted.

6D here, it would be important to supplement the mRNA data with protein data.

7,8 : I am not an expert in ALS, but the beneficiary effects of enoxacin appear modest at best. Would it be possible to compare the effects to a drug that is already in use, as discussed by the authors? Again, it was difficult to follow the statistical analysis without a detailed presentation in the figure legends. Datasets 7F and G are highly variable, and significance of the results is questionable.

1st Revision - authors' response 12 May 2015

Point By point response to referees:

Referee #1:

Emde et al find evidence for altered microRNA processing in motoneurons of ALS patients and in ALS in vitro systems. Pharmacological enhancement of miRNA processing restores some of the

phenotypes, but does not prolong the life of SOD1 mice. The changes in miRNA levels upon various genetic and pharmacological manipulations are attributed to stress granule (SG) formation, but stress granules are never directly analyzed throughout the whole manuscript. The authors postulate an interesting new hypothesis and show data in various model systems (including an impressive two mouse models). On the one side, it is nice that miRNAs seem to be affect different ALS models, on the other side there is no red thread throughout the manuscripts. The authors switch between genetic and pharmaceutical induction of different kinds of cellular stress and analyze the effect on a changing set of miRNAs. Several connections are implied, but not firmly shown. To justify publication in the EMBO journal the
mechanistic links between ALS mutations, stress granules and miRNA processing needs to be carved out more carefully.
Eran: We thank the referee for an overall positive response.

Specific points:
Fig 1A: The global suppression of microRNA expression is stunning. The effect depends on the validity of two reference genes. The authors should show qPCR data for other unrelated small RNAs (e.g. snoRNAs) normalized to the same reference RNAs.
Eran: The ABI miRNA PCR arrays contain six "reference genes" RNU6B, RNU24, RNU43, RNU44, RNU48 and mammU6. Of these six, we originally chose nucleolar RNU48 (SNORD48) and splicosome-related U6 (mammU6) for their robust expression. We now added RNU44 (SNORD44) for normalization as well, which did not change the overall result. In Clark's column RNU44 levels were below detection levels and hence we normalized Clark's column RNA expression to only RNU48 and mammU6. Three additional RNAs (RNU6B, RNU24, RNU43) were at borderline detection levels in many of the samples and hence omitted. Unfortunately, from the original samples, analyzed by Ravits and Moeller more than 5 years ago, there are no leftovers to go back to. We therefore tested other non coding RNAs in a new set of samples that Ravits is now investigating. Thus, we tested 27 long ncRNAs and these were similarly expressed in another cohort of 12 non-ALS and 12 sporadic ALS nervous systems, with Pearson correlation of 0.9882. RNA from autopsies was isolated after laser capture microdissection of lumbar motor neurons. The 27 long ncRNAs tested were MALAT1, TSIX, XIST, MIAT, SNHG1, EMX2OS, DLEU2, H19, HAR1A, PART1, PVT1, ATXN8OS, HOTAIR, SNHG6, HESRG, HULC, PRINS, PCGEM1, UCA1, HAR1B, GHRLOS, DISC2, BPESC1, BCYRN1, LOC554202, PCA3, KCNQ1OT1. This data set is part of unpublished work of John Ravits et al. Therefore, miRNA downregulation is notable, in comparison to other non-coding RNAs in human ALS motor neurons.

It would be nice to see global changes in expression of pre-miRNA and mature miRNAs by P32 5' end-labeling.
Eran: We were unsuccessful in establishing 5'-labeling protocol on spinal cord punches from SOD1 G93A mice. We regret that this is the case and started developing a protocol that seems to be able to complement the data presented in the current manuscript. It is a RNA-sequencing protocol for characterizing miRNAs in motor neurons that are isolated by laser-capture microdissection from SOD-1 G93A lumbar motor neurons at four different age groups (Day 30, 90, 120, 137). This extensive effort will take time that does not allow us to include it as part of the current manuscript.

Fig 1 E/F: Are the miRNA changes in sALS and fALS correlated? The raw data for Fig 1 should be included as a table.
Eran: We provide tables for all data (Supplementary table 1). We measured the correlation of changes in individual miRNAs in sALS and fALS and the Pearson correlation value is 0.3.

Fig 2: Why were these miRNAs selected? Why are different miRNAs shown in the different panels.
Eran: As in a similar reply to a comment from referee #2: We picked specific miRNAs that were expressed at different levels. We chose representative miRNAs that are expressed in human and mouse motor neurons including, miR-10b, miR-30a, miR-103, (abundantly expressed in isolated mouse motor neurons), miR-218, miR-30c, miR-138 and let-7b (highly expressed in human motor neurons); miR-132, let-7c, let-7d (neuronal, mid-range in human); miR-143, let-7a (low expression in human neurons). In the revised work, we increased the uniformity of the miRNA species in different panels. Panels now repeatedly include a coherent set of 12 pre-miRNA and mature miRNA pairs (Figures 2,3,4,5,7 and Appendix Figures S2, S4, S5, S7, S8, S10).

Do different stressors affect different miRNAs? If so, how?

Eran: There is literature referring to effect of different stressors on specific miRNAs. For example: Byrd et al., 2012 J Cell Biol 196:689-98 or El Azzouzi et al., 2013 Cell Metab. 18:341-54. However, we characterized a common behavior, prefer to stay with the key phenomenon and thus not to extend the work towards deviating variants.

The legend (grey vs. black) should be clearly shown in all graphs
Eran: We add textual notes into the panels throughout the manuscript.

Fig 2j/k: Quantification of several replicates needs to be shown.
Eran: Extended Western blot analysis is shown now in all figures with quantified densitometry analysis. See Figures 2, 5 and in Appendix Figures: S1, S3, S6, S8, S10).

Fig 2k is not mentioned in the text. Are DICER levels reduced by arsenite treatment?
Eran: DICER levels are not reduced after arsenite treatment (Figure 2M).

Fig 2L: What's known?
Eran: The data on Puromycin was moved to Figure 7. Puromycin effect on miRNA biogenesis was not investigated before.

Fig 2L: Puromycin inhibits overall translation. Are the protein levels of the DICER complex components reduced to directly inhibit DICER activity independent of stress granules?
Eran: We performed analysis of protein levels for TRBP, AGO2, PACT and DICER that is now moved to Appendix Figure 6 related to Fig 7. Puromycin in indeed known to inhibit translation and DICER and TRBP levels are reduced by 50% and 30%, respectively. Yet, DICER activity is reduced even without reduction of any of the DICER complex components, for example, when cultures are treated with Thapsigargin or Paraquat (Figure 2). All these conditions were sufficient for stress granule formation (Figure 6). Our interpretation is that DICER activity is reduced by many stressors, whereas DICER protein level is unchanged under most conditions used in this work with the exception of Puromycin.

Compare slight effect on DICER by arsenite treatment in Fig. 2K. The authors claim the change in miRNAs is due to induction of stress granules.
Eran: DICER levels are not reduced after Sodium arsenite treatment (Figure 2M), while inducing stress granule formation (Figure 6). Therefore, our interpretation is that down regulation of DICER activity is observed upon conditions that are sufficient for stress granule forming, even without reduction of any of the DICER complex components.

The authors should show that puromycin really induces stress granules within the 24h by immunofluorescence (ideally time course experiment).
Eran: We provide immunofluorescence analysis to show that Puromycin induces stress granules at two different concentrations (Figure 6).

Fig 3A: Does FUS R495X expression induce cellular stress and cause formation of stress granules in this model?
Eran: We provide immunofluorescence analysis to show that FUS R495X induces stress granules (Figure 6). In the text we added that it may be that high plasmid copy number and the stress of the transient transfection contributed to induction of SGs by of wild-type FUS, which was not reported in a stably-transfected cell lines (Bosco et al, 2010; Daigle et al, 2013 ; Dormann et al, 2010).

In most publications mutant FUS localizes to SGs only after stress induction with arsenite or heat shock (e.g. Bosco et al 2010, Dormann et al 2010, Daigle et al 2013) but not by itself. The discrepancy may be due to different stress levels induced by the various transfection protocols. The authors should confirm that only transfection of mutant FUS does indeed induce SGs under their conditions using immunofluorescence with marker antibodies.
They should also show the effect of mutant FUS compared to wildtype FUS on at least on the most regulated miRNAs.
Eran: We performed extensive additional analysis. Under our transfection protocol, both wild-type or mutant FUS were sufficient to generate stress granules (Figure 6). Noteworthy, at least one other study reported the localization of acutely transfected wild-type FUS into stress granules (Andersson et al., 2008 The multifunctional FUS, EWS and TAF15 proto-oncoproteins show cell type-specific

expression patterns and involvement in cell spreading and stress response. BMC Cell Biol 9:37). A plausible interpretation is that the transient transfection provides additional required stress. However, transfection of GFP-expressing vector was not sufficient to drive stress granule formation, suggesting that wild-type FUS has innate propensity to drive stress granule formation when overexpressed and given acute/ transient transfection stress. In the text, we added a comment about the differences of our transfection method and those used in previous reports.

Also, why are some miRNAs more affected? Does this reflect a different half-life time?
Eran: This is an important question that will require additional analysis outside the scope of the manuscript. For revisions, we investigated the potential correlation between previously-reported miRNA species half-life time from Guo et al., 2015 Nucl. Acids Res. 43: 2326-2341 'Characterization of the mammalian miRNA turnover landscape'. We were unable to identify sequence rules that explain the trend behavior of specific miRNAs in our samples.

Fig 3C: It seems that mutant FUS reduces DICER protein levels. Please show quantification.
DICER, AGO2, PACT and TRBP levels are unchanged after overexpression of wild-type FUS or R495X FUS, as seen in a quantitative Western blot analysis in Supplementary Figure 3 related to Figure 3.

Fig 4A: The authors need to show that mutant protein expression indeed induces SGs specifically compared to wt proteins by IF and quantify miRNA changes also compared to wt control. Especially overexpression the wildtype RNA-binding proteins TDP-43 and FUS could by itself affect miRNA levels.
Eran: The referee suggested an important set of experiments. Via performing three different assays, namely, Dicing assay, qPCR analysis of pre-miRNA / mature miRNA levels and confocal immunofluorescence study of stress granules we learn that the impact of wild-type protein overexpression, is reminiscent of their respective mutant counterparts. Thus, TDP-43 (wild-type of A315T) and FUS (wild-type or R495X) induce stress granules, concomitant with reducing DICER activity (Figures 3, 4, 6 and Appendix Figure S2). Overexpression of wild-type SOD1 or a SOD1 G93A mutant also reduced Dicing, although to lesser extent and it also initiated stress granules. We discuss these observations in the 'results' and 'discussion' sections of the revised manuscript and suggest that TDP-43 and FUS may affect Dicing more dramatically potentially because these are RNA-binding proteins that are identified in SGs.

Moreover, they have to show that CHX treatment inhibits SG formation and not merely reduces expression of the mutant proteins. I don't know the half-life time of these proteins, but I could imagine that 72h CHX treatment would dramatically reduce expression levels of the transfected proteins. This would severely confound interpretation of this rescue experiment.
Eran: The low dose Cycloheximide protocol we have used (CHX, 0.02 µg/ml), did not reduce the levels of exogenous GFP-FUS-R495X or FLAG-TDP-43-A315T, as shown in Supplementary Figure 8. We performed immunofluorescence study to show that this CHX treatment still reduced SG formation (Fig 7 G,H) and accordingly recovered DICER activity (Figure 7).

What are the effects of CHX on miRNA levels in untransfected cells?
Eran: miRNAs levels are unchanged in untransfected cells, treated with CHX (0.02 µg/ml), which is now shown in Appendix Figure S8 related to Figure 7.

Fig 4B,C: The authors should validate their key findings by western blot.
Eran: We performed IPs and Western blot studies that are presented in Fig. 5D. AGO2 interactions with PCBP1 was verified. Upregulation of AGO2 binding to DICER was twofold and verified by two independent MS protocols when either AGO2 or DICER were immuneprecipitated, yet Western analysis was borderline. We were unable to validate increased interactions of AGO2 and PACT or DICER.

Fig 4D,E: These experiments should also be performed with wt control.
Eran: We removed these panels from the new version due to technical difficulties in measurements.

Fig 4A,F and 5: error bars missing
Eran: All data are displayed with error bars.

Fig 5: error bars missing
Eran: Statistics are revised. All data are displayed with error bars.

Fig 6A/B: I would prefer to see how the miRNAs and mRNAs levels are changing in the control mice of time instead of normalization to 1.
Eran: As requested by the referee, we present data in Appendix Figure S10 with normalization only to the initial day of measurements in wild-type tissue (day 77).

Fig 6C: Are these the most dysregulated miRNAs in SOD1 animals?
Eran: To streamline of our analysis we picked the same miRNAs that are also presented in Figures 2,3,4,5,7 and Appendix Figure S2, S4, S5, S7, S8. We did not rank the miRNAs relative to other miRNAs.

Fig 6D: How the expression levels of miRNA processing machinery in SOD1 mice compared to nontransgenic littermates?
Eran: These are unchanged and presented in Appendix Figure S10. qPCR analysis of mRNA and Western blot analysis of protein levels.

Fig 7F,G,H,J: No post-test was done in the ANOVA. Are the relevant pairs not significantly different?
Eran: All ANOVA now complemented by Holm-Sidak post-tests.

Fig 7i: Here a post-test was performed, but the relevant pairs should be marked in the graph.
Eran: We improved visualization of pair-wise statistical comparison in this case and throughout all other figures.

Fig 7j: Does a lower hangwire score indicate greater strength or were the labels swapped?
Eran: Lower hangwire score indicate greater strength; we modified the legend accordingly.

Fig 7K: How are two curves compared by a t-test?
Eran: We revised the statistics using ANOVA, now presented in Figure 8K.

Referee #2:

In the present manuscript, Emde et al. explore a potential role of impaired pre-miRNA processing in ALS. They show that stress and ALS mutations lead to altered pre-miR/mature miR levels for some miRNAs, possibly by affecting the composition of the DICER processing complex and eIF2alpha signaling. Treatment of mice with an enhancer of miR processing, Enoxacin, provides benefitial effects for some neurological aspects of ALS. Invoking altered miR processing in ALS is a novel and interesting hypothesis, and the elucidation of such a mechanism could provide a novel avenue for ALS treatment. However, the data presented in this manuscript is in most cases at best preliminary and largely does not support the claims of the authors. Before this manuscript can be published, it has to be extensively revised, i.e. by completing preliminary datasets, by providing new experiments that functionally link stress-dependent remodeling of the DICER complex to altered miRNA biogenesis in ALS and by providing a sound statistical analysis.

Eran: We extensively revised the work in line with the request of referee #2. I believe that the new version addresses most of the general concerns. We are providing new experiments that functionally link stress-dependent remodeling of the DICER complex to altered miRNA biogenesis: (1) Confocal fluorescent micrographs depicting co-localization of AGO2 into stress granules under diverse set of stressors or upon over-expression of ALS-causing genes (Figures 6, 7). (2) Direct induction of stress granules via overexpression of stress granule proteins TIAR, EIF5A or with phosphomimetic form of EIF2A (S51D) drives AGO2 to stress granules (Figure 6), alongside with downregulation of DICER activity (Figure 5). (3) Furthermore, cell free Dicing assay directly demonstrated reduction in DICER activity under stress conditions or with overexpression of ALS-causing proteins, stress granule nucleating protein TIAR or with EIF5A (Figures 4, 5, 7).

In addition, we streamlined measured miRNAs and increased the uniformity of the miRNA species depicted in different panels investigate and we have revised the statistical analysis. We introduced

bar graphs with error bars for standard error of the mean and describe the statistical method used in each and every figure legend.

In addition, the authors should pay attention to my detailed comments provided below.

General comments:

1. The data presentation, in particular graphs supposed to show altered pre-miRNA processing, are very confusing. Why are the authors not presenting individual bars for pre-miRNAs and miRNA levels under different treatment conditions, instead of ratios?
Eran: we now provide individual bars for pre-miRNA and miRNA levels and all data are displayed with error bars and statistical analysis. In order to make data easier to grasp, we define an *Inhibition score*, which is the ratio of pre-miRNAs to mature miRNAs and normalized the value calculated for individual miRNA species to the corresponding control sample value. This value is >1 when DICER activity is reduced. In Figure 2 we present individual bars and *Inhibition score*. Later we present the *Inhibition scores* in Figures 2, 3, 4, 5, 7 and the corresponding individual measurements in Appendix Figures (S2, S4, S5,S7,S9).

2. The statistical analysis is completely insufficient. Some panels lack error bars entirely, why in others different time points are grouped together to achieve statistical significance. Moreover, statistical methods are not presented in the figure legends, making an independnet assessment oft he data impossible. For example, often single bars are marked with asterisks, without any indication to what other value(s) the comparison was actually made (e.g. Fig. 2, 4). If the data were normalized, how could a statistical significance actually be calculated?
Eran: These points were well taken and we have revised the statistics thoroughly. All panels possess error bars. Statistical methods are now presented in the figure legends. All asterisks (denoting p-values) possess graphical annotation pointing the data compared. In analysis of animal studies, we performed ANOVA with post-hoc Holm-Sidak test, where applied.

3. The rationale for choosing miRNA candidates is not provided. Were these the miRs which displayed the most pronounced differences?
Eran: As in the comment to referee #1: We picked specific miRNAs that were expressed at different levels. We chose representative miRNAs that are expressed in human and mouse motor neurons including, miR-10b, miR-30a, miR-103, (abundantly expressed in isolated mouse motor neurons), miR-218, miR-30c, miR-138 and let-7b (highly expressed in human motor neurons); miR-132, let-7c, let-7d (neuronal, mid-range in human); miR-143, let-7a (low expression in human neurons). In the revised work, we increased the uniformity of the miRNA species in different panels. Panels now repeatedly include a coherent set of 12 pre-miRNA and mature miRNA pairs (Figures 2, 3, 4, 5, 7 and Appendix Figure S2, S4, S5, S7, S8, S10).

There is also quite some discrepancy regarding the results between different cellular models (i.e. HEK cells vs. Motoneurons), but this point was not addressed.
Eran: In discussion, we refer to discrepancy regarding the results between different cellular models. On page 15 it reads:" However, the variability gained with different stressors, or by using different cell types suggests additional factors that are currently unknown. Furthermore, the effect of SOD1 on DICER activity is weaker than the effect of RNA-binding proteins TDP-43 and FUS that were reported in SGs. Therefore, it may be that RNA-binding proteins are phenotypically more potent than SOD1 in driving dysregulation of miRNA biogenesis."

4. Although altered pre-miR/miRNA ratio correlate with changes in the composition of the DICER complex, there is actually no data that definitely shows reduced DICER activity. One solution would be to perform in vitro DICER processing assays with extracts obtained from the different models. This would also allow to deplete/substitute components of the DICER complex to interrogate their potential contribution to altered pre-miR processing.
Eran: This is a key point of the revised manuscript and we thank the referee for asking us to establish a Dicing assay in cell lysates. We developed an *in vitro* system for evaluating changes in DICER activity using cell lysates and an exogenous substrate. The new assay was used to reveal downregulation of DICER activity in data shown in Figures 4B, 4C, 4D, 5E, 7B.

5. Overall, the recovery obtained with Enoxacin treatment appears to be rather modest, and only a few specific miRNAs seem to be affected (Fig. 5C).

Eran: The new data set provided in the revised version as part of Supplementary 10D we show significant effects of Enoxacin on 4/12 miRNAs and three more with marginal significance, whereas no miRNA of the 12 was downregulated. Given the degree of noise when RNA is extracted from the whole motor cortex tissue and not from isolated motor neurons, we think these data are reassuring in the context of the overall analysis and support the focus on DICER as a novel pathway in stress and in ALS.

There is also a disconnect in that Enoxacin targets the DICER-TRBP interaction, which is not affected by stress (Fig. 4C). This raises the question whether enoxacin is in fact the right drug to improve miRNA processing in ALS.
Eran: DICER complex activity depends on several co-factors and is reduced under stress. miRNA biogenesis in cultured cells or in the brain tissue is accordingly responsive to Enoxacin (Figures 4, Supplementary Figure 10). The beneficial effect of Enoxacin on miRNA biogenesis in the brain was studied also in Front Psychiatry. 2014; 5: 6. doi: 10.3389/fpsyt.2014.00006; PMC3918929. Enoxacin is a particularly intriguing candidate for clinical assessment, based on its established safety and pharmacokinetic profile, however, more effective DICER agonists might exhibit in the future, when these are developed, more dramatic beneficial effect in ALS.

Specific concerns:
2A-I: The presentation of pre-miR vs. Mature miR is very confusing. The authors should show values for pre-miR and mature miR for both control and experimental condition.
Eran: we now provide individual bars for pre-miRNA and miRNA levels and all data are displayed with error bars and statistical analysis. Based on the qPCR results for individual mature miRNAs and pre-miRNAs we defined an "*Inhibition score"* , which is the ratio of pre-miRNAs to mature miRNAs and is set to a value of "1" in control and is >1 when DICER activity is reduced . In Figure 2 we present individual bars and *Inhibition scores*. We believe that this ratio is easier to grasp, and hence we present *Inhibition scores* in Figures 2, 3, 4, 5, 7 and the corresponding measurements of pre-miRNAs and mature miRNAs in Appendix Figures (S2, S4, S5, S7, S9).

How are the p-values actually calculated? This should be clearly indicated in the figure legends.
Eran: we now clarify statistical approaches in the figure legends.

There is also no discussion how miR candidates were selected, and why the processing of only a specific subset is significantly affected. In this context, the authors should tone down some of their statements regarding global effects on miR processing.
Eran: We picked specific miRNAs that were expressed at different levels. We chose representative miRNAs that are expressed in human and mouse motor neurons including, miR-10b, miR-30a, miR-103, (abundantly expressed in isolated mouse motor neurons), miR-218, miR-30c, miR-138 and let-7b (highly expressed in human motor neurons); miR-132, let-7c, let-7d (neuronal, mid-range in human); miR-143, let-7a (low expression in human neurons). In the revised work, we increased the uniformity of the miRNA species in different panels. Panels now repeatedly include a coherent set of 12 pre-miRNA and mature miRNA pairs (Figures 2,3,4,5,7 and Appendix Figure S2, S4, S5, S7, S8, S10).

We toned down statements regarding global effects on miRNA processing in the discussion of variability between experimental conditions: "The variability in miRNA biogenesis with different stressors, or by using different cell types suggests additional factors that are currently unknown"; also for discussion of Enoxacin:" However, more effective Dicing activity agonists might exhibit more dramatic beneficial effects in ALS in the future" and for the variability between data on SOD1 and TDP-43, FUS: "Furthermore, SOD1 effect on DICER activity may be more limited than the effects mediated by FUS or TDP-43, which are RNA-binding proteins that are directly engaged in SGs."

2K: From this blot, it looks like DICER is strongly downregulated upon stress, but this observation is neither discussed nor followed up. It would also be important to show the full blots to see other DICER isoforms that have been reported.
Eran: We respect EMBO J policy and provide full blots in a separated source data file. Figure 2K is now replaced by extended analysis including densitometry and statistical analysis (Figure 2M). Since the expression levels of the four proteins in the DICER complex were not down-regulated by two independent stressors (Thapsigargin, Paraquat), in the time and doses that were sufficient to

inhibit Dicing, our interpretation is that alteration of DICER activity may occur even without reduction in DICER complex components.

2L: what is this figure supposed to show? Most of the data is quite variable and differences do not reach statistical significance. Moreover, puromycin clearly affects processes other than stress granule formation, which should be discussed.
Eran: To better connect the context we positioned the panel on Puromycin in Figure 7 along with the CHX study, as it reads better this way. We repeated the experiments and have now improved statistics that are shown in Figure 7C, and Supplementary Figure 7A,B. 8/12 pre-miRNA and 7/12 mature miRNAs are changes in statistically significant manner. Puromycin clearly affects processes other than stress granule formation, but when considered with several other lines of investigation, we think it supports our claim about SGs. For example, it is unlikely that the effect of Puromycin was due to a general inhibition of protein synthesis since CHX, which equally inhibits translation, had an opposite effect (Fig. 7B). Additional new experiments with direct overexpression of SG proteins are more further support this view.

3A: Now, even a different form of data presentation compared to Fig. 2 is chosen, which leads to further confusion. The fact that a substantial fraction of miRs actually shows reduced pre-miR/miR ratios is not discussed.
Eran: Based on the Editor's guide to streamline the work and because the sequencing analysis was technically very challenging and fragile, we decided to take this analysis out of the current manuscript version. We believe it makes the interpretation simpler.

4A: These panels lack error bars or p-values, which makes it impossible to judge the reproducibility of the experiments.
Eran: Statistics are revised. All data are displayed with error bars.

Since cycloheximide is a blocker of translation, other translation blockers should be used for comparison, e.g. emetine.
Eran: We have used Puromycin, another inhibitor of translation and further moved the two data sets adjacently so flow becomes more natural (New Figure 7). Phosphomimetic EIF2A, a molecular cue for inhibition of translation that like Puromycin results in SG assembly (micrographs in Figure 6) inhibits Dicing (Figure 5G, Supplementary Figure 5C,D vs. Figure 7B,C and Supplementary Fig. 7A,B).

4B,C: It would be important to show representative blots.
Eran: We validated key aspects of mass spectrometry study by western blot in Figure 5D.

Since these experiments are done in HEK cells, which show quite different effects on miR processing compared to motoneurons, the relevance of these findings remains somewhat unclear.
Eran: We emphasize in the discussion why we think that downregulation of DICER activity is commonly observed under stress in many cell lines: "The sensitivity of DICER activity to cellular stress is not limited to motor neurons and may be probably relevant to other tissues under chronic stress, where decrease in miRNA or DICER abundance was reported (Inukai & Slack, 2013; Kaneko et al, 2011; Mori et al, 2012; Nidadavolu et al, 2013). However, the variability gained with different stressors, or by using different cell types suggests additional factors that are currently unknown. Furthermore, the effect of SOD1 on DICER activity is weaker than the effect of RNA-binding proteins TDP-43 and FUS that were reported in SGs. Therefore, it may be that RNA-binding proteins are phenotypically more potent than SOD1 in driving dysregulation of miRNA biogenesis."

As mentioned above, it is quite puzzling that the major regulatory proteins of DICER, TRBP and PACT, are not affected by stress.
Eran: We did not observe downregulation of DICER or its cofactors, under the following stress conditions: Paraquat, Thapsigargin, FUS wild-type and FUS R495X mutant. The simplest interpretation that we prefer is that there downregulation of DICER activity is possible without changes in protein expression levels. Sodium arsenite upregulated DICER (171%) and downregulated TRBP and PACT (both to 66% of the expression in the control); For Puromycin, the processing defect might be explained merely by downregulation of DICER and TRBP (to 46%, 69% of the expression in the control). It is plausible that some of the chemicals (especially if applied at higher doses than those we used) will generally block translation and drive reduction of protein

synthesis and DICER complex levels. However, under the experimental conditions we used, and with the exception of Puromycin, inhibition of DICER activity is observed without change in cofactor levels.

4D: again, the presented changes are apparently not significant, so what is this figure supposed to show? Statistical test is not indicated.
Eran: We took this analysis out of the manuscript, as these changes were not significant.
4F: see my comments to Fig. 4A
Eran: We took this analysis out of the manuscript, as these changes were not significant.
5: see my comments to Fig. 4A and F.
Eran: we now provide individual bars for pre-miRNA and miRNA levels with Enoxacin treatment in Supplementary Figure 4A-F and all data are displayed with error bars and statistical analysis. *Inhibition score* before or after Enoxacin treatment, namely the ratio of pre-miRNAs to mature miRNAs is shown in Figure 4E-G with error bars for s.e.m and significant p-values are indicated by asterisks. Processivity of individual miRNA species is normalized to the corresponding control sample value and value of >1 reflects reduced DICER activity relative to control.

6A, B: now, a different panel of miRs was interrogated. I found it quite unusual that significance was calculated over different time points, so this should be complemented by a statistical assessment of individual time points. Also, the test used should be indicated.
Eran: These data are now in Supplementary Fig. 10A. We streamlined the panel of miRNAs interrogated according to all other figures. Statistical assessment is of individual time points. We removed data on days 90 and 120 and focus on comparing days 77 and 137, for both changes overtime and comparing directly wild-type to mutant at specific time points.

6C: This figure actually shows that enoxacin has only very modest effects on the relative expression of miRs, and that only a minority of miRs is affected. This should be clearly stated in the text.
Eran: In the new Supplementary Fig. 10D we performed the required measurements in the brains of SOD1 G93A mutant mice without or with Enoxacin treatment. Significant effects were demonstrated in 4/12 miRNAs and three more with marginal significance. Given that molecularly SOD1 impact is more limited than TDP-43 or FUS (Fig. 3, Appendix Figure S2) and the degree of noise when RNA is extracted from the whole motor cortex tissue and not from isolated motor neurons, we think these data are reassuring in the context of the overall analysis.

In addition, an important control experiment (control vs. Control enoxacin) that addresses the effects of enoxacin in control animals was omitted.
Eran: Control vs. control Enoxacin is added into New Supplementary Figure 10E.

6D here, it would be important to supplement the mRNA data with protein data.
Eran: Western blot analysis in New Supplementary Figure 10C reveals that levels of AGO2, PACT, TRBP and DICER are unchanged in SOD1 G93A mouse spinal cords.

7,8 : I am not an expert in ALS, but the beneficiary effects of enoxacin appear modest at best. Would it be possible to compare the effects to a drug that is already in use, as discussed by the authors?
Eran: The G93A model we used is the harshest SOD1 mouse model. It is in fact a notoriously aggressive transgene. E.g., despite established efficacy of Riluzole in human ALS patients, a recent work using failed to reveal any effect in the SOD1 G93A mouse model (Li et al., PLoS One. 2013 8:e65976). We elaborate on these issues in the revised discussion also plays downs the statement to say that it validates DICER as new therapeutic target.
"…We did not observe any lifespan extension, but also Riluzole, the only FDA-approved drug for ALS, lacks survival benefit in SOD1 G93A mice (Li et al, 2013a; Scott et al, 2008). Therefore, as previously suggested (Scott et al, 2008), the predictive value of the aggressive SOD1 G93A model may be limited in testing novel pharmacological interventions. Furthermore, SOD1 effect on DICER activity may be more limited than the effects mediated by FUS or TDP-43, which are RNA-binding proteins that are directly engaged in SGs.
Nonetheless, if DICER and miRNAs are indeed instrumental in the pathogenesis of ALS in humans, as we suggest, these observations hold promise for future intervention by validating DICER as new therapeutic target. Furthermore, Enoxacin is a particularly intriguing candidate for clinical assessment, based on its established safety and pharmacokinetic profile and because several forms

of ALS converge in the down-regulation of miRNAs. However, more effective Dicing activity agonists might exhibit more dramatic beneficial effects in ALS in the future.
Again, it was difficult to follow the statistical analysis without a detailed presentation in the figure legends. Datasets 7F and G are highly variable, and significance of the results is questionable
Eran: statistics are revised in New Figure 8F,G. The analysis indeed reveals subtle effect as the referee said. ANOVA of Four-paw comparison is significant. Post-Hoc Holm-Sidak tests did not reveal significant differences comparing single paw behavior.

2nd Editorial Decision 09 June 2015

Thank you for submitting your revised manuscript to the EMBO Journal. Your study has now been re-reviewed by the two referees and their comments are provided below. As you can see, the referees appreciate the introduced changes and support publication in The EMBO Journal. I am therefore very pleased to accept the manuscript for publication here.

REFEREE REPORTS

Referee #1:

The authors address my main points and the extensively revised manuscript is now ready for publication in EMBO J.

Referee #2:

The authors have satisfactorily addressed most of my previous concerns and the paper is now ready for publication

